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INTESTINAL MICROFLORA OF THE LARVAE OF ST. MARK'S FLY

IV. STUDIES ON THE INTESTINAL BACTERIAL FLORA OF A LARVA-POPULATION

By

I. Szabó, Maria Marton and Ilona Buti

Institute for Soil Research and Agrochemistry (Director: I. Szabolcs*) of the Hungarian Academy of Sciences, Budapest*

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Summary. In the intestinal tract of the larvae of St. Mark's fly there is a definite selection of bacterial flora ingested with organic remains and soil matter. The absolute predominant member of the bacterial population comprising more than 70% of the total intestinal microflora was *Pseudomonas fluorescens*. *Enterobacter aerogenes* occurring infrequently in soil and an intermediary *Enterobacteriaceae* biotype were also important constituents. Some representatives of the soil microflora (*Bacillus cereus*, *Mycobacterium* sp., *Chromobacterium* sp., etc.) survived the intestinal passage and were recovered in more or less reduced numbers. Others, mainly inhabitants of horizons A_H and A_F (*Bacillus subtilis*, *Bacillus sphaericus*, *Alcaligenes faecalis*, *Achromobacter pestifer*, *Agrobacterium radiobacter*, *Rhizobium* spp., *Micrococcus cryophilus*, etc.) were absent from the middle and hind intestine and from fresh excrement. Although this type of selection is characteristic, the qualitative and quantitative composition of the predominant organisms is considerably influenced by environmental factors (food and soil), which may alter the survival chance of soil microflora passing through the digestive canal.

Computer analysis of the intestinal actinomycete flora of *Bibio marci* larvae revealed complicated selection processes taking place in the digestive canal [1]. It has been shown that only some species of the actinomycete population in soil are able to multiply in the intestinal tract of the larvae. In view of the fact that actinomycetes play only a secondary role in the intestine of St. Mark's fly, it seemed important to study these selection processes in relation to enteric bacteria. The investigations were performed on individual and collective intestinal samples taken from 220 larvae of population No. 6 described in our previous paper [2].

Materials and methods

1. *Quantitative examination of intestinal bacterial flora* was performed with specimens taken aseptically from the middle and hind intestine and with fresh excrement by use of the following media: (a) N-free Ashby agar; (b) peptone agar; (c) casein-glucose agar; (d) glucose-asparagine agar; (e) cellulose-peptone agar; (f) cellulose ammonium sulphate agar; (g) glycerolarginine agar. Media (e) and (f) were incubated both acrobically and anaerobically.

2. *Isolation of strains* for identification was performed by transferring colonies from the above media to agar slant (see below).

3. *Pure culture* was obtained by streaking from the stock culture or from dilution series made on peptone-glycerol agar onto plates. Reisolation of the strain was checked by streaking

simultaneously on agar plates. During the examination period the purity of the strains was checked by making smears and plate cultures [3].

4. *Maintenance of strains* was made partly by freeze-drying partly by storing at 4—6°C. Strains maintained on slants were subcultured at intervals of 4 days to 2 weeks according to the viability of the cultures. After inoculation the media were incubated at 28°C for 24—28 hours.

5. *Standard inoculum*. A loopful of culture grown on the stock agar medium for 24—48 hours, or 0.05 ml of one loopful of bacteria suspended in 10 ml saline was used.

6. *Stock agar medium*. Most strains grew well on yeast-extract-glucose agar [4]; yeast extract, 5 g; peptone, 5 g; glucose, 10 g; agar, 20 g; distilled water, 1000 ml; pH 7.2.

7. *Incubation*, except for certain special examinations, was performed at 28°C.

8. *Colony morphology*. Peptone-glycerol agar, stock agar medium and sometimes special media were used. The properties were recorded after 2—6 days incubation as follows: (a) colony type (S, SR, R); (b) edge (undulate, rhizoid, dentated, lobated); (c) elevation (flat, papillary, wrinkled, convex); (d) consistency (butyrous, dry-friable, mucoid, viscid); (e) surface (glistening, matt).

9. *Cell morphology*. (a) Arrangement of cells (filament, chain, cluster, packet); (b) shape according to HARRINGTON's classification [5]: long rods, $> 5\ \mu$; medium rods, $3\ \mu - 5\ \mu$; short rods, $< 3\ \mu$; coccus; oval; branching; (c) presence of spores.

10. *Examination of cell morphology* was performed with 24—48 hour cultures grown on stock agar medium under phase-contrast microscope and in Gram-stained smears. Motility was examined in hanging drops prepared with 2 day peptone water cultures. The method of GEMMEL and HODGKISS [6] elaborated for the macroscopic detection of the motility of lactobacilli was also employed.

11. *Flagella staining and electron microscopic studies*. Flagellae were stained by PEPPLER's tannin method. Electron micrographs were prepared in the Tesla 242A apparatus. Agar medium was overlayered with collodion, seeded by spraying the bacterial suspension on the membrane and incubated until microcolonies developed. Then the membrane was mounted and shadowed with palladium at 20° angle.

12. *Spores* were shown by staining with aniline fuchsin then with methylene blue.

13. *Cultural characters*. In comparing the cultures pigment production on peptone-glycerol agar, dissociation on starch agar medium, etc. were considered. For the detection of characteristic pigments of certain organisms special media were employed.

14. *Growth characters* were examined on peptone-glycerol agar slants incubated at 37°C and 45°C for 48 hours and at 5°C for 1 week.

15. *Heat tolerance*. Twenty-four hour cultures prepared in nutrient broth (meat extract, 3.0 g; peptone, 5.0 g; distilled water, 1000 ml) were exposed in water bath to 56°C and 60°C for 10 minutes. The cultures were then rapidly cooled, subcultured on stock agar slants, incubated for 48 hours and compared with the unheated control.

16. *Influence of pH on growth*. Nutrient broth was adjusted to pH 4.0 with HCl and to 8.0 with NaOH. Observation of the inoculated medium lasted for 6 days.

17. *Salt tolerance* was tested in nutrient broth containing 3.5 and 10% NaCl. Observation lasted for 6 days.

18. *Phenol resistance* was tested as described by SNEATH [7]. Aliquots of 1.6 ml nutrient broth culture were incubated for 48 hours at 28°C. Then 0.4 ml 5% aqueous phenol solution was added to each tube. After exposure at 20°C for 7 minutes 0.05 ml samples were diluted with 5 ml broth. Finally one drop of the suspension was transferred to agar slant and incubated for 4 days.

19. *Indole production*. Three to 6 day peptone water (Bacto peptone, 1%; NaCl, 0.5%; pH 7.2) cultures were extracted with xylene and tested with KOVÁCS's reagent.

20. *Ammonia production* was detected in 4 day peptone water cultures with Nessler reagent.

21. *Arginine hydrolysis*. After incubation for 5 days in arginine-glucose medium (arginine, 3 g; glucose, 1 g; K_2HPO_4, 1 g; yeast extract, 1 g; distilled water, 1000 ml; pH 7.0) ammonia was shown with Nessler reagent.

22. *Arginine dihydrolase* was detected as described by RICHARD [8].

23. *Hydrogen sulphide* production from cysteine, sodium thiosulphate and peptone was shown with lead acetate paper strips. Culture media for the detection of H_2S production from cysteine and thiosulphate consisted of: glycerol, 12 ml; asparagine, 1.5 g; K_2HPO_4, 1.5 g; NaCl, 3.0 g; $MgCl_2$, 0.75 g; $CaCO_3$, 0.3 g; distilled water, 1500 ml. To 1.5 litres of basal medium 0.185 g thiosulphate or 0.283 g L-cysteine was added.

24. *Methyl red* (MR) and *Voges—Proskauer* (VP) reactions were tested in fluid glucose-phosphate medium. Acetoin was detected by BARRIT's method.

25. *Nitrate reduction* was examined in ISP nitrate broth (Difco). Nitrite was shown after 4—12 days incubation with Griess—Ilosvay reagent. Reduction beyond nitrite was tested by the zinc dust method.

26. *Starch hydrolysis* was detected with Lugol solution after 5 days incubation. Basal medium: peptone, 5.0 g; meat extract, 5.0 g; NaCl, 5.0 g; agar, 15.0 g; soluble starch, 4.0 g; distilled water, 1000 ml;

27. *Aesculin hydrolysis* was tested by SNEATH's method [7] in peptone-aesculin-iron citrate medium.

28. *Urease activity* was shown on CHRISTENSEN's agar [9] incubated for 2—7 days.

29. *Lecithinase activity* was tested on egg-yolk agar.

30. *Cellulase activity* was detected by microscopic examination of filter paper strips incubated for 7—14 days in peptone water or ammonium sulphate liquid medium.

31. *Methylene blue reduction.* Basal medium: meat extract, 3 g; tryptone, 5 g; distilled water, 1000 ml. After 24 hours incubation 1 drop of 1% methylene blue solution was added and the medium was incubated further for 1—24 hours.

32. *Proteolytic activity.* Nutrient gelatin stabs and LIESKE's egg-white agar plates [11] were incubated for 17 days. Activity on egg-white agar was expressed as the radius of clear zones.

33. *Haemolytic activity.* Basal medium: meat extract, 5.0 g; peptone, 10.0 g; NaCl, 3.0 g; K_2HPO_4 2.0 g; agar, 17.0 g; distilled water, 1000 ml. To each plate 1 ml defibrinated sheep blood was added. Incubation lasted for 2—4 days.

34. *Antibiotic sensitivity.* Peptone-meat extract agar plates were seeded with 24—48 hour cultures, then the antibiotic discs were placed on the plates and after 24 hours incubation the radius of inhibition zones was measured. The following discs manufactured by the Institute for Serobacteriological Production and Research "Human", Budapest, were used: penicillin (3 IU), oxacillin (10 μg), methicillin (20 μg), chloramphenicol (30 μg), streptomycin (30 μg), oleandomycin (30 μg), tetracycline (30 μg), neomycin (100 μg), polymyxin-B (15 μg), erythromycin (10 μg), superseptyl (sulphonamide derivative, 400 μg), nitrofurantoin (300 μg), chlortetracycline (30 μg), oxytetracycline (30 μg), vancomycin (50 μg), kanamycin (30 μg), spiramycin (30 μg), novobiocin (30 μg).

35. *Oxidative and fermentative breakdown* of glucose and lactose was tested by the method of HUGH and LEIFSON [12].

36. *Oxidase test.* The method of KOVÁCS [13] was used.

37. *Catalase production.* Ten per cent hydrogen peroxide was added to 24—48 hour agar slants and gas production was recorded. An uninoculated slant served as control.

38. *Production of growth inhibitory substances.* The test organisms were seeded on peptone-meat extract agar (peptone, 5.0 g; meat extract, 5.0 g; NaCl, 5.0 g; distilled water, 1000 ml; pH 7.0) and the antagonist strain was either spotted or transferred as agar block culture onto the plate. Test organisms: *E. coli*, *B. subtilis*, *Saccharomyces carlsbergensis*.

39. *Tyrosinase activity.* Basal medium: L-tyrosine, 1.0 g; yeast extract (Difco), 1.0 g; NaCl, 8.5 g; agar, 16.0 g; tap water, 1000 ml. Incubation lasted for 24—48 hours.

40. *Casein hydrolysis.* Basal medium: meat extract, 3.0 g; tryptone, 5.0 g; glucose, 10.0 g; agar, 15.0 g; distilled water, 1000 ml; pH 7.0; skim milk, 0.5 ml/Petri dish. Incubation lasted for 2—3 days.

41. *Citrate utilization* was tested on SIMMONS' solid medium.

42. *Lipase activity.* SIERRA's method [14] with Tween-80 substrate was used.

43. *Ornithine decarboxylase* was tested according to the method of RICHARD [8]. Lysine decarboxylase activity was demonstrated as described by MØLLER [15].

44. *Phenylalanine deaminase.* The method of COLLINS [16] was used.

45. *Acid production* from D-galactose, maltose, sucrose, L-arabinose, D-fructose, D-mannitol and glycerol was tested in the medium of HUGH and LEIFSON [12].

46. *Carbohydrate utilization as sole source of carbon.* Basal medium [17]: $(NH_4)_2SO_4$, 2.64 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Difco agar, 20 g; distilled water, 980 ml; pH 7.0; the medium was sterilized by autoclaving at 115°C for 30 minutes. The carbon sources (glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol, inositol, glycerol) were Seitz filtered and added at 0.5% final concentrations. The basal medium was used as control. Incubation lasted for 16 days.

47. *Utilization of nitrogen sources.* Basal medium [17]: KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; glucose, 3.0 g; glycerol, 2.0 g; Difco agar 20 g; distilled water, 1000 ml; pH 7.0. Nitrogen sources ($NaNO_3$, glycine, L-cystine, L-tryptophan, L-arginine and L-asparagine) were added so that the final nitrogen concentration corresponded to 280 mg per litre. The cultures were inoculated also on nitrogen-free Ashby agar.

Results

From intestinal and excrement specimens seeded on Ashby agar no anaerobic or aerobic nitrogen fixing bacteria were isolated. The results were also negative for anaerobic cellulose-decomposing bacteria. On cellulose agar incubated aerobically only some fungi were occasionally observed. These findings confirmed our previous results [18] that in the intestine of larvae there is neither increase in absolute nitrogen content of the organic material ingested nor a significant decomposition of cellulose. The predominating intestinal organisms require less complex nutrient substances, most of them grow well even on inorganic nitrogen sources, or belong to lactose-splitting *Enterobacteriaceae*. Comparison of colonies on primary plates and properties of the isolates indicated that about 2/3 of the bacterial flora belonged to one single species. Seventy out of 100 strains isolated from the intestinal milieu represented this species. Its importance in respect to the ecology of *Bibio* larvae is clearly understood if it is considered that in mixed gut-content specimens from larval community No. 6 the incidence of the bacteria was higher than 70% of the total intestinal flora. These organisms were Gram negative non-acid-fast and non-sporulating rods producing sometimes filaments, were briskly motile by two or more polar flagella, produced green fluorescent pigment and failed to attack lactose. In Table I three representatives of pseudomonads isolated from the intestine of the larvae (strains B-044, B-067 and B-068) are compared to authentic strains. Strains B-067 and B-068 represented predominant pseudomonads of the digestive canal; B-044 was a representative of a frequent variant. These bacteria corresponded to organisms described as "*Ps. fluorescens* species group" [19] or as "*Ps. fluorescens* species" [20]. Strains *Ps. fluorescens* CCM 1969 (NCIB 3756) was very similar in characters to strains B-067 and B-068 (Table I).

Reactions and intensity of growth were denoted in Table I in two different manners, namely on the one hand as + = positive, (+) = doubtful or weakly positive, − = negative reaction or no growth, on the other as − = negative, 1 = weak, 2 = medium, 3 = strong reaction or abundant growth. The degree of antibiotic sensitivity was recorded similarly. Abbreviations: S = smooth, R = rough, Lo = lobular, F = flat, Wr = wrinkled, Bu = butyrous, Ge = glistening, M = matt, r = limit of opaque zone is not sharp, Al = alkaline reaction. The incidence of pseudomonads in the intestine of lower animals is known from the literature [1]. The high incidence in the intestinal tract of *Bibio* larvae reflects the uniformity of the bacterial flora as it has been observed for actinomycetes. *Ps. aeruginosa* strain CCM 1959 used for comparison was able to grow even at 45°C and showed a strict physiological relationship with LÁNYI's general description of this species [21] based on a large number of isolates. The systematic position and individual species rank [20] of *Ps. ovalis* have not yet been elucidated.

Table I

Comparison of Pseudomonas cultures isolated from the digestive canal of the larvae of St. Mark's fly with authentic strains

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
Peptone-glycerol agar						
Colony type	S	S	S	S	R	R
Edge of colony	Lo	Lo	Lo	Lo	(Lo)	Lo
Elevation of colony	F	F	F	F	Wr	F
Consistency	Bu	Bu	Bu	Bu	Bu	Bu
Surface	Ge	Ge	Ge	Ge	M	M
Soluble pigment	—	—	—	—	—	—
Colour of colony	—	—	—	—	—	—
Green fluorescent pigment	+	+	+	+	—	+
Oxidase (Kovács)	3	3	2	2	3	3
Lecithinase	3r	1	3r	3r	1	3
Lipase (Tween-80)	—	—	3	3	—	3
Aesculin hydrolysis	—	—	—	—	—	—
Starch hydrolysis	—	—	—	—	—	—
Casein hydrolysis	2	1	3	3	2	2
Arginine hydrolysis	3	±	3	3	3	3
Ammonia from peptone	3	1	3	3	3	1
Citrate (Simmons)	1, Al	1, Al	1, Al	1, Al	1, Al	1, Al
Haemolysis	1	1	2α	—	1	1
Litmus reduction	+	—	+	+	—	+
Phenylalanine deaminase	—	—	—	—	—	—
Taurocholate	3	3	3	3	3	3
Acid production						
D-Galactose	+	+	+	+	+	+
Maltose	—	—	—	—	—	—
Sucrose	—	—	—	—	—	—
L-Arabinose	+	+	+	+	+	+
D-Fructose	(+)	—	—	—	—	—
D-Mannitol	—	+	—	—	—	(+)
Glycerol	(+)	—	—	—	—	—
Resistance to phenol	—	—	—	—	—	—
Heat tolerance, 56—60°C	—	—	—	—	—	—
pH tolerance, 4.0	—	—	—	—	—	—
8.0	3	3	3	3	3	3

Table I continued

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
NaCl tolerance 3.5%	3	1	3	3	2	1
10.0%	−	−	−	−	−	−
H_2S production						
Cysteine	1	3	−	−	−	−
$Na_2S_2O_3$	−	−	−	−	−	−
Peptone	−	−	−	−	−	−
Tresner–Danga reaction	−	−	−	−	−	−
Gram staining	−	−	−	−	−	−
Ziehl–Neelsen staining	−	−	−	−	−	−
Spores	−	−	−	−	−	−
Motility	+	+	+	+	+	+
MR	−	−	−	−	−	−
VP	−	−	−	−	−	−
Indole	−	−	−	−	−	−
Proteolytic activity						
Gelatin	−	−	−	1	−	2
Egg-white agar	−	−	1	1	−	1
Cellulase	−	−	−	−	−	−
Nitrate reduction						
to nitrite	+	−	+	+	−	+
beyond nitrite	+	−	+	+	−	+
Glucose breakdown						
oxidative	+	+	+	+	+	+
fermentative	−	−	−	−	−	−
Lactose breakdown						
oxidative	−	−	−	−	−	−
fermentative	−	−	−	−	−	−
Methylene blue reduction	3	2	3	3	3	3
Urease	3	±	3	3	2	3
Growth 5°C	3	2	3	3	2	−
37°C	±	±	3	3	± − 1	3
45°C	−	−	−	−	−	2
Inhibition of the growth of						
E. coli	−	−	−	−	−	−
B. subtilis	−	1	1	1	1	2
Sacch. carlsbergensis	1	2	2	2	1	3

Table 1 continued

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
Catalase	3	3	3	3	3	3
Tyrosinase	—	—	—	—	—	—
Antibiotic sensitivity						
Penicillin	—	—	—	—	—	—
Oxacillin	—	—	—	—	—	—
Methicillin	—	—	—	—	—	—
Chloramphenicol	±	±	—	—	—	—
Streptomycin	1	1	1	1	1	1
Oleandomycin	—	—	—	—	—	—
Tetracycline	1	1	1	1	1	1
Neomycin	1	1	1	1	1	1
Polymyxin-B	1	1	1	1	1	1
Erythromycin	—	—	—	—	—	—
Superseptyl	1	1	1	1	±	—
Nitrofurantoin	—	—	—	—	—	—
Chlortetracycline	1	1	1	1	1	1
Oxytetracycline	1	1	1	1	1	1
Vancomycin	—	—	—	—	—	—
Kanamycin	1	1	1	1	1	1
Spiramycin	—	—	—	—	—	—
Novobiocin	—	—	—	—	—	—
Carbon source utilization						
Glucose	1	1	1	1	1	1
Fructose	1	1	1	1	1	1
Arabinose	—	1	—	—	—	—
Rhamnose	—	—	—	—	—	—
Xylose	—	—	—	—	—	—
Galactose	1	1	1	1	1	1
Maltose	—	—	—	—	—	—
Mannitol	1	2	—	—	—	1
Inositol	±, 1	—	—	—	—	—
Glycerol	1	2	1	1	1	1
Negative control (without C)	—	—	—	—	—	—
Positive control (peptone)	3	3	3	3	3	3
Nitrogen source utilization						
$NaNO_3$	2	—	2	2	—	1

Table I continued

	Ps. fluorescens CCM 1969 (NCIB 3756)	*Ps. fluorescens* B-044	*Ps. fluorescens* B-067	*Ps. fluorescens* B-068	*Ps. ovalis* CCM 1977 (ATCC 8209)	*Ps. aeruginosa* CCM 1959 (NCIB 6750)
Glycine	2	3	3	3	2	2
L-Cystine	—	2	—	—	—	—
L-Tryptophan	1	—	—	—	—	—
L-Asparagine	2	3	2	2	2	2
L-Arginine	2	3	3	3	2	2
Negative control (without N)	—	—	—	—	—	—

In lack of suitable methods the percentage incidence of other species of the intestinal flora was not determined. However, we give an account of the approximate frequency of these organisms. *Enterobacter* (Aerobacter) *aerogenes* occurred much less frequently than *Ps. fluorescens*. The properties of *E. aerogenes* strain B-014 chosen as a representative of the isolates were as follows: $0.3-0.6 \times 0.5-1.4\,\mu$ motile rods arranged singly or in pairs, sometimes in short filaments and chains. Electron micrography indicated the presence of peritrichate flagellae. IMViC reaction $--++$ (characteristic of the species); oxidase, lecithinase, lipase, phenylalanine deaminase, tyrosinase, cellulase, proteolytic activity and starch hydrolysis negative; casein hydrolysed weakly; nitrates reduced to nitrite; methylene blue reductase, catalase and urease positive; oxidative and fermentative breakdown of glucose and lactose; hydrogen sulphide produced from cysteine, peptone and sodium thiosulphate [1]; Tresner–Danga negative; intense ammonia production from peptone and arginine; weak haemolysis; aesculin rapidly hydrolysed; litmus not reduced; milk strongly acidified; abundant growth and acid production on MacConkey agar; acid production from D-galactose, maltose, L-arabinose, D-fructose, D-mannitol, glycerol, but not from sucrose. Killed at 56°C, not resistant to phenol; growth at pH 8.0 but not at 4.0; 3.5% NaCl well tolerated but no growth at 10% NaCl; growth at 5°C and 37°C but not at 45°C; arginine dihydrolase positive, no dehydrogenation, nucleus or chain breakdown of steroids; growth of *B. subtilis* and *Sacch. carlsbergensis* inhibited but no effect on *E. coli*. Resistant to penicillin, oxacillin, methicillin, oleandomycin, erythromycin, superseptyl, vancomycin, spiramycin and novobiocin, moderately sensitive to chloramphenicol, streptomycin, tetracycline, neomycin, polymyxin-B, nitrofurantoin, chlortetracycline, oxytetracycline, and kanamycin. Utilizes glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol

but not inositol as sole source of carbon. Utilizes well glycine, L-asparagine and L-arginine, weakly L-cystine but not L-tryptophan and $NaNO_3$ as nitrogen source. Produces on peptone-glycerol agar S-type, mucoid, colourless colonies with undulate edge and glistening surface; produces no soluble pigment; Gram negative, non-sporulating, non-acid-fast rods.

In addition to *E. aerogenes* an as yet unidentified organism comprised a characteristic although less frequently occurring member of the intestinal flora. The representative strain, B-061, showed the following properties: glucose rapidly fermented; lactose not attacked aerobically or anaerobically; IMViC test — (+) — —; oxidase, lecithinase, lipase, phenylalanine deaminase, tyrosinase, cellulase, proteolytic activity negative; casein and starch hydrolysed; weak catalase activity; nitrate reduced to nitrite; urease negative; methylene blue weakly reduced; hydrogen sulphide produced from cysteine but not from sodium thiosulphate and peptone; Tresner—Danga negative; ammonia not produced from peptone or arginine; haemolysis nil; aesculin rapidly hydrolysed; litmus milk unchanged; good growth but no acid production on MacConkey agar; acid produced from D-galactose, maltose, sucrose, and glycerol but not from L-arabinose, D-fructose and D-mannitol. Resistant to phenol and tolerates 56°C but not 60°C, grows at pH 8.0 but not at 4.0; tolerates 3.5% but not 10% NaCl; grows well at 5°C but not at 37°C; no dehydrogenation, nucleus or chain breakdown of steroids; exerts no definite antibiotic effect; resistant to nitrofurantoin, weakly sensitive to penicillin, oxacillin, methicillin, oleandomycin, polymyxin-B, erythromycin, kanamycin and spiramycin; moderately sensitive to chloramphenicol, streptomycin, tetracycline, neomycin, superseptyl, chlortetracycline, oxytetracycline, vancomycin and novobiocin; grows only on complex media and fails to utilize glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol, inositol and glycerol as carbon source; fails to grow at the expense of inorganic nitrogen and to utilize amino acids as nitrogen sources; on peptone-glycerol agar produces easily emulsifiable S-type colonies light yellow in colour with undulate edge and flat glistening surface; forms no soluble pigment, produces no spores, Gram negative and non-acid-fast. This organism belonged neither to *Escherichia* nor to *Enterobacter*. We regard it as an intermediary organism of an as yet undetermined taxonomic position. In Fig. 1 it is designated as "Paracolobactrum".

In addition to organisms discussed above, 4 different species of bacteria were encountered. From their constant presence in low numbers it has been concluded that — being common inhabitants of soil — they are relatively resistant to the selective effect of intestinal passage and their populations pass through the intestine more or less unchanged and without playing any important role. Isolates of this group of organisms belonged to *Bacillus cereus* [22]. The characters of the culture representing these isolates (strain B-062) and of authentic *B. cereus* cultures are shown in Table II.

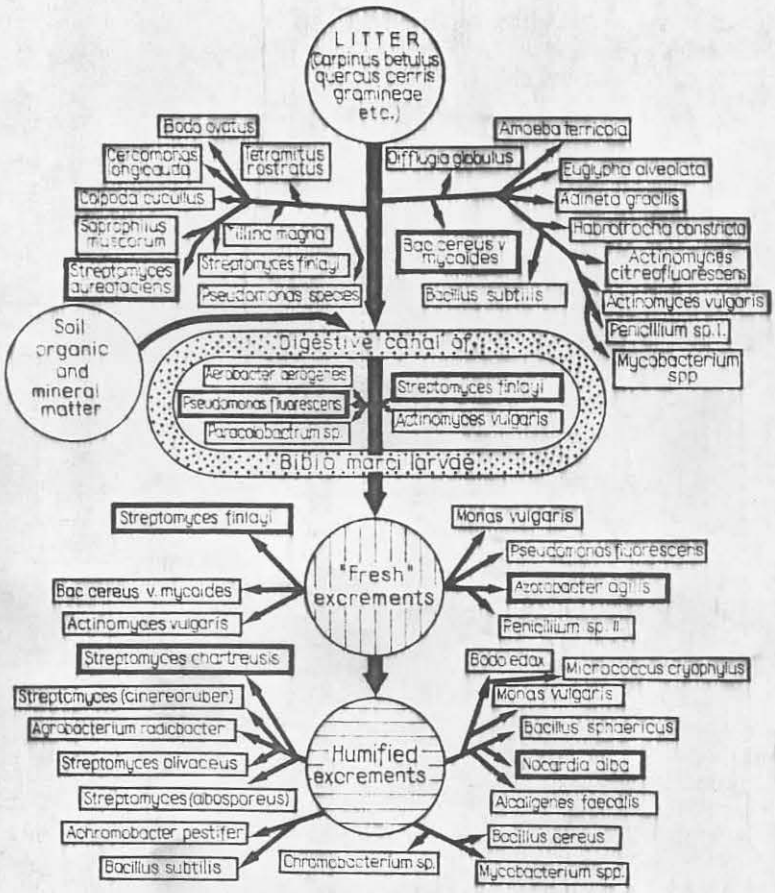

Fig. 1. **Succession of microbial communities as an effect of a *Bibio marci* larva-population in the Förna of a mull like forest rendzina**

It is evident that strain B-062 belongs to the species *B. cereus*. It is interesting that *B. cereus* var. mycoides occurred frequently in the soil but was absent from intestinal and excrement specimens.

Strains belonging to one *Mycobacterium*, one *Chromobacterium* and one *Brevibacterium* species were isolated occasionally. The *Chromobacterium* strains were not identical with *C. violaceum* and *C. lividum* described by SNEATH [7] and MOFFET and COLWELL [23]. As these organisms occur in horizon A_H and even in the rhizoplane of certain plants, they will be discussed in the general description of the bacterial flora of rendzina soil [24].

As the identification of many soil mycobacteria presents extreme difficulties, only the characters of strain B-047, representative of the *Mycobacterium* species isolated from the intestinal specimens are given: glucose and lactose are attacked neither oxidatively nor fermentatively; oxidase, lecithinase, phenylalanine deaminase, tyrosinase, cellulase negative; lipase positive; no

Table II

Comparison of Bacillus cereus strains

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
Peptone-glycerol agar				
Colony type	R	R	R	R
Edge of colony	(Un)	(Un)	(Un)	Rh
Elevation of colony	F	F	F	F
Consistency	Dr. sm.	Dr. sm.	Dr. sm.	Dr. sm.
Surface	M	M	M	M
Soluble pigment	– (yellow)	– (yellow)	– (yellow)	– (yellow)
Colour of colony	–	–	–	–
Oxidase (Kovács)	–	–	–	–
Lecithinase	1	1	–	?
Lipase (Tween-80)	2	2	1	2
Aesculin hydrolysis	3	2	3	2
Starch hydrolysis	3	3	3	3
Casein hydrolysis	2	2	2	2
Arginine hydrolysis	–	–	–	3
Ammonia from peptone	±	1	±	3
Citrate (Simmons)	–	± – 1. Al	3. Al	–
Haemolysis	3β	2β	3β	1
Litmus reduction	+	+	+	+
Phenylalanine deaminase	–	–	–	–
Taurocholate	–	–	–	–
Acid production				
D-Galactose	–	(+)	–	–
Maltose	+	(+)	+	+
Sucrose	(+)	–	–	–
L-Arabinose	–	–	–	–
D-Fructose	(+)	+	+	+
D-Mannitol	–	–	–	–
Glycerol	–	–	–	(+)
Resistance to phenol	3	3	3	3
Heat tolerance, 56°C	3	3	3	3
60°C	3	3	3	3
pH tolerance, 4.0	–	–	–	–
8.0	3	2	2	3

Table II continued

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
NaCl tolerance, 3,5%	2	2	2	3
10%	–	–	–	–
H_2S production				
Cysteine	3	3	3	–
$Na_2S_2O_3$	–	–	–	–
Peptone	2	–	–	1
Tresner—Danga reaction	–	–	–	–
Gram staining	+	+	+	+
Spores	+	+	+	+
Motility	+	–	+	–
MR	(+)	+	–	–
VP	(+)	+	(+)	–
Indole	–	–	–	–
Proteolytic activity				
Gelatin	3	3	2	2
Egg-white agar	1	2	1	3
Peptonization of milk	+	+	+	+
Cellulase	–	–	–	–
Nitrate reduction				
to nitrite	+	+	+	+
beyond nitrite	–	–	–	–
Glucose breakdown				
oxidative	+	–	+	?
fermentative	+	+	+	?
Lactose breakdown				
oxidative	–	–	–	–
fermentative	(+)	(+)	(+)	–
Methylene blue reduction	3	3	–	2
Urease	–	–	–	–
Growth 5°C	2	–	–	3
37°C	1	3	2	3
45°C	–	1	±	–
Inhibition of the growth of				
E. coli	–	–	–	–
B. subtilis	1	–	1	–
Sacch. carlsbergensis	±	1	±	±

Table II continued

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
Catalase	3	3	3	3
Tyrosinase	—	—	—	—
Antibiotic sensitivity				
Penicillin	—	—	—	1
Oxacillin	—	—	—	1
Methicillin	2	2	2	1
Chloramphenicol	1	1	1	1
Streptomycin	1	1	1	1
Oleandomycin	1	1	1	1
Tetracycline	2	2	2	2
Neomycin	1	1	1	1
Polymyxin-B	1	1	1	1
Erythromycin	1	1	1	1
Superseptyl	1	2	1	1
Nitrofurantoin	1	1	1	1
Chlortetracycline	2	2	2	2
Oxytetracycline	2	2	2	2
Vancomycin	1	1	1	1
Kanamycin	1	1	1	1
Spiramycin	1	1	1	1
Novobiocin	2	2	2	2
Carbon source utilization				
Glucose	±.1	±.1	±	±
Fructose	—	±.1	—	—
Arabinose	—	—	—	—
Rhamnose	—	—	—	—
Xylose	—	—	—	—
Galactose	—	—	—	—
Maltose	±.1	±.1	±	±
Mannitol	—	—	—	—
Inositol	—	—	—	—
Glycerol	—	—	—	—
Negative control (without C)	—	—	—	—
Positive control (peptone)	3	3	3	3
Nitrogen source utilization				
$NaNO_3$	—	—	—	—

Table II continued

	B. cereus B-062	*B. cereus* OKI 100 002 (IP 5257)	*B. cereus* RIPP, A-115	*B. cereus* var. mycoides RIPP, A-111
Glycine	±	2	1	2
L-Cystine	–	–	–	–
L-Tryptophan	–	–	–	–
L-Asparagine	1	2	1	1
L-Arginine	1	2	1	±
Negative control (without N)	–	–	–	–

proteolytic activity; casein and starch not hydrolysed; catalase positive; nitrate reduced to nitrite; urease positive; methylene blue reduction weak; hydrogen sulphide produced from peptone and cysteine but not from sodium thiosulphate; Tresner—Danga negative; ammonia production from peptone weak, from arginine nil; weak haemolysis; traces of aesculin hydrolysis; litmus milk unchanged; abundant growth and acid production on MacConkey agar; acid production from D-fructose, weak acid from D-mannitol; D-galactose, maltose, sucrose, L-arabinose and glycerol not attacked; resistant to phenol; heating at 60°C tolerated; rapid growth at pH 8.0, no multiplication at pH 4.0; 3.5% NaCl tolerated, no growth at 10% NaCl; steroids dehydrogenated, nucleus and chains attacked; antibiotic activity exerted only against yeasts; resistant to oxacillin and methicillin, moderately sensitive to oleandomycin, polymyxin-B, nitrofurantoin, spiramycin, novobiocin, sensitive to penicillin, chloramphenicol, streptomycin, tetracycline, neomycin, erythromycin, superseptyl, chlortetracycline, oxytetracycline, vancomycin and kanamycin; among carbon sources glucose, fructose, mannitol and glycerol utilized well, galactose weakly and rhamnose, arabinose, xylose, maltose and inositol not at all; good growth in defined medium with $NaNO_3$ and different amino acids (glycine L-cysteine, L-tryptophan, L-arginine, asparagine); no growth on Ashby agar; on peptone glycerol agar R-type, at first emulsifiable then dry pink colonies with undulate edge; no soluble pigment; non-sporulating Gram positive, moderately acid-fast rods 0.5—0.7 × 0.5—8.0 μ in size arranged singly, in pairs or in short chains. Strain B-047 belongs to TSUKAMURA's group B of mycobacteria [17]. According to the scheme of CERBON and BOJALIL [25] it falls into "Branch 1" of rapidly growing orange pigment-producing mycobacteria. Of *M. smegmatis*, *M. phlei* and "irregular branch" comprising the above unit, strain B-047 belongs to the latter. It is interesting that in comparing *M. phlei*, *M. smegmatis*, *M. fortuitum* and *M. rhodochrous*, GORDON and MIHM [26] found that only *M. smegmatis* and *M. fortuitum* produced

changes in the colour of MacConkey agar (in 43 and in 96%, respectively). Strain B-047 grew well on MacConkey agar and produced acid.

Finally the description of strain B-057 contributes to data for *Brevibacterium*. The culture, not belonging to any known species of the genus, was characterized as follows: attacks glucose and lactose neither fermentatively nor oxidatively; oxidase, phenylalanine deaminase, tyrosinase, cellulase and lipase negative; lecithinase weakly positive; proteolytic activity and casein hydrolysis negative; hydrolyses starch; catalase positive; nitrate reduction negative; urease negative; reduces methylene blue rapidly; produces hydrogen sulphide from cysteine and peptone but not from sodium thiosulphate; Tresner—Danga negative; produces ammonia from peptone in slight amounts but not from arginine; weak haemolysis; rapid aesculin hydrolysis; reduces litmus; grows on MacConkey agar but fails to produce acid; produces acid from D-galactose, maltose, L-arabinose, D-fructose and glycerol but not from sucrose and D-mannitol; moderately resistant to phenol and tolerates 56°C; grows at pH 8.0 but not at pH 4.0; highest level of NaCl tolerance 3.5%; fails to dehydrogenate steroids and to attack the nucleus and chains; exerts no antibiotic effect; resistant to penicillin, oxacillin, superseptyl, nitrofurantoin, spiramycin; moderately sensitive to methicillin, streptomycin, oleandomycin, polymyxin-B, erythromycin and kanamycin; sensitive to chloramphenicol, tetracycline, neomycin, chlortetracycline, vancomycin and novobiocin; grows only on complex media; fails to utilize inorganic nitrogen sources and amino acids; does not grow in the presence of glucose, fructose, arabinose, rhamnose, xylose, galactose, maltose, mannitol, inositol and glycerol as sole sources of carbon; no growth on N-free Ashby agar; on peptone-glycerol agar produces easily emulsifiable, colourless colonies with undulate edge and glistening surface; produces no soluble pigment; Gram positive, non-acid-fast, non-sporulating rods 0.3—0.5 × 0.8—4 μ in size arranged singly or in pairs.

The examination of intestinal and excrement specimens confirmed that bacterial species predominant in, and characteristic of, horizons A_H, A_F and A_{00} [24] occur in small numbers or not at all in the intestinal tract of *Bibio* larvae. Thus *B. subtilis*, *B. sphaericus*, *Alcaligenes faecalis*, *Achromobacter pestifer*, *Agrobacterium radiobacter*, *Rhizobium* spp., *Micrococcus cryophilus*, etc., the common inhabitants of rendzina milieu were entirely absent from the fresh excrement of the animals.

The present results, as mentioned in the introduction, reflect the examination of intestinal and excrement specimens taken from larva-population No. 6 [2]. It seemed desirable to perform an orientation analysis in other larva-populations of the rendzina milieu. These examinations will be described in a subsequent paper. However, it is necessary to point out here that the selection of microflora taking place in the intestine can generally be demonstrated, but as to systematic position and quantitative relationship the bacterial species

gaining predominance in the intestine may vary considerably. For the difference many factors as quality of food, physiological state of the animal, composition of soil microflora, etc., may be responsible.

Discussion

The present studies on the intestinal bacterial microflora of *Bibio* larvae indicate a selection similar in mechanism to the selection of intestinal actinomycetes [1, 27]. In course of the intestinal passage of litter and soil matter certain microorganisms, though they may be ingested in very small numbers, become predominant members of the animals' flora. These bacteria are able to multiply rapidly in the digestive canal and play an important role in the metabolism of the animal and also in an at least partly elimination of other microorganisms ingested with the food. The characteristic representatives of the intestinal flora in *Bibio* larvae belong to the families *Pseudomonadaceae* and *Enterobacteriaceae*. It may be supposed that the intestinal microflora shows a certain degree of qualitative and quantitative difference not only in the imagos and larvae of various insect species, but also in populations of the same species living in different environments. The alteration may not affect only the predominant bacteria but also those that survive the intestinal passage ("additional intestinal flora elements"). Fig. 1 shows bacterial species predominant in the intestine of larvae of larva-population No. 6 and in the environment. The sketch is a supplemented form of a scheme prepared on the basis of our previous zoological-bacteriological investigations [28]. The arrows indicate the direction of the material and energy flow. The predominant members of microflora and fauna developing in the litter composed mainly of the remainders of *Carpinus betulus*, *Quercus cerris* and Gramineae are as follows: *Amoeba terricola*, *Bodo ovatus*, *Colpoda cucullus*, *Difflugia globulus*, *Saprophilus muscorum*, *Actinomyces citreofluorescens*, *Streptomyces aureofaciens*, *Penicillium* spp., *Bacillus cereus* var. mycoides, *Bacillus subtilis*, *Mycobacterium* spp., *Agrobacterium* spp., etc. These organisms metabolize directly or indirectly the organic matters of the litter. They and other organisms contaminating the soil matter in the upper layer of horizon A_H are ingested by *Bibio* larvae. During intestinal passage the total bacterial count increases to manyfold, while the number of species decreases. In the digestive canal, where bacteria always gain an absolute predominance, actinomycetes are represented by *Streptomyces finlayi* and small numbers of *Actinomyces* (Streptomyces) *vulgaris*. The predominating bacterial species are *Ps. fluorescens*, *E. aerogenes*, intermediary *Enterobacteriaceae* strains, etc. Protozoa and rotifera [29] characteristic of the litter disappear completely. In the excrement actinomycetes (first of all *Str. finlayi* common also in the intestine) become predominant in a week after excretion and increase their number to 80% of the total flora. Certain bacteria

also multiply in the excrement to high numbers (*Azotobacter agilis*, *B. cereus* var. mycoides); yet, *Ps. fluorescens* still occurs very frequently. In a few days or weeks the microflora of the fresh excrement changes into a population characteristic of the "humified" excrement: many streptomycetes including the predominant *Str. chartreusis* and locally accumulating *Nocardia alba*, as well as a wide variety of bacteria, yeasts and protozoa. The scheme shown in Fig. 1 represents, of course, only one of the many possible combinations which may develop in different environments.

Further studies on the intestinal microflora of *Bibio* larvae will be presented in subsequent papers.

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Address of the authors:
ISTVÁN SZABÓ, MARIA MARTON, ILONA BUTI,
Institute for Soil Research and Agrochemistry of the Hungarian Academy of Sciences, Herman O. út 15, Budapest II, Hungary